The role of the Common Fragile Site Gene product, Fhit, in protection from DNA damage

Senior Honors Research Thesis

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By

Jessica Bene

The Ohio State University

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Project Advisor: Dr. Kay Huebner, Department of Molecular Virology, Immunology, and Medical Genetics

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Abstract

Genomic instability is a fascinating hallmark of cancer, important for the accelerated acquisition of cancer-driving mutations. The causes and consequences of genomic instability are not fully understood and have been the subject of intensive investigation by many laboratory groups. The fragile site gene, FHIT, located in locus FRA3B, encodes a protein that demonstrates tumor suppressive functions, is one of the earliest targets mutated in cancer, and has been hypothesized to be a "guardian of the preneoplastic genome" (Pichiorri et al. 2008) To investigate the role of Fhit in promoting genomic integrity, Fhit function was examined in multiple cell lines including cancerous, non-cancerous, and mouse tissue-derived cells. From these studies it was determined that Fhit acts to prevent DNA double-strand breaks (DSBs). Experimental evidence suggests that Fhit utilizes its dinucleoside substrate–binding activity to suppress DSBs, and Fhit mutants with abolished substrate-binding activity fail to minimize DNA breaks. Further study of Fhit positive and negative cells with different types of genotoxic agents suggested that Fhit prevents DNA replication stress, and that replication stress is the source of DNA breaks in Fhit-negative cells. The mechanism(s) through which Fhit participates in this function is still unknown. In experiments in which Chk1, ATR, or ATM were inhibited, each of which are kinases central in the cellular response to replication stress and DSBs, it was found that Fhit and Chk1 functions to prevent DSBs were co-dependent. This functional interaction indicates that Fhit and Chk1 act directly or indirectly within the same pathway during replication stress. From these findings, coupled with the fact that the FHIT gene is one of the earliest and most frequently altered targets in cancer, it is concluded that the loss of Fhit in precancerous cells can therefore contribute to cancer initiation due to the development of DNA breaks and genomic instability. The discovery that the Fhit protein is essential for genome stability is important because it shows that the Fhit loss observed in early preneoplastic lesions underlies the initiation of the genomic instability that

is the Hallmark of cancer development and progression. Future experiments designed to understand how Fhit performs this ‘caretaker’ function will allow identification of pathways that may become cancer prevention or therapy targets.

Introduction

Cancer is one of the most studied diseases in the United States and indeed the world. There are various types of this disease such as stomach cancer, lung cancer, lymphoma, breast cancer, and leukemia to name a few. It arises from cells growing and dividing without regulation, to form benign or malignant tumors. Before the formation of a tumor, the cells are termed "preneoplastic" but once the tumor, or the abnormal tissue growth, is present the cells are termed "neoplastic." Tumors categorized as "malignant" are those that can invade and metastasize to other tissues and organs and are responsible for the majority of cancer-related deaths.

Cancer cells most commonly develop through mutations in one of two types of genes, oncogenes or tumor suppressor genes. Oncogenes promote cell proliferation, invasion, and metastasis, while tumor suppressor genes repress these processes. Mutations may either cause overexpression or hyperactivation of oncogenes, or they may cause reduced expression or inactivation of tumor suppressor genes.

Genomic instability is considered a "hallmark" of cancer. It is characteristic of almost all types of cancer (Negrini 2010). There are different forms of genomic instability, including chromosomal instability and microsatellite instability. Genomic instability facilitates tumorigenesis by increasing the likelihood of acquiring mutations or alterations of expression of oncogenes and tumor suppressor genes. Hereditary cancers often express mutations in DNA repair genes as well, which in turn causes genomic instability (for review, Kinzler & Vogelstein, 1997). However, the majority of cancers are not hereditary and do not have mutated DNA repair genes, yet they still develop genomic instability. Some activated oncogenes expressed in precancerous lesions and cancer have the potential to induce DNA double strand breaks (DSBs) activating the DNA damage response (DDR) (Halazonetis et al. 2008). Activation of the DDR is

a key distinguishing characteristic of precancerous and cancerous lesions from normal cells. Precancerous cells must bypass the DDR barrier in order to form tumors.

When activated, certain oncogenes induce replication stress, cause DNA DSBs and chromosomal instability at genomic loci referred to as "common fragile sites" (CFSs). CFSs, which are present in all individuals, are large genomic regions that are prone to instability, seen as breaks and gaps in the metaphase chromosomes. CFSs can be seen in metaphase chromosomes of normal white blood cells that have been cultured in conditions of replication stress (Pichiorri et al. 2008). These sites show an extreme sensitivity to replication stress and when exposed to this stress, incur small and large deletions. CFSs are also hotspots for sister chromatid exchange and translocations (Durkin et al. 2006).

Why CFSs are sensitive to replication stress has been extensively studied, and some similarities have been found between the different fragile sites in the genome. CFSs do not exhibit similarities in DNA sequence, but most fragile sites are rich in AT basepairs (Huebner et al. 2001). It has also been suggested that fragile sites possess clusters of sequences with high flexibility and low stability, possibly contributing to fragility to replication stress. It has also been reported that there is a link between fragile site instability and late replication time, leading to the hypothesis that delayed replication might cause the fragility at replication stress-induced fragile sites, and the gaps and breaks seen in these sites are most likely incompletely replicated DNA. Later it was indicated that common fragile site chromatin expressed histone hypoacetylation which may suggest that this more compact chromatin structure may play a role in the genomic instability at the fragile site regions (Saldivar et al. 2010).

The most fragile CFS was found to be FRA3B, located at 3p14.2 (Ohta et al. 1996; Zimonjic et al. 1997) overlapping the FHIT gene (Huebner et al. 2001). Alterations in FHIT have

been associated with preneoplastic cells, benign tumors, and malignant tumors in many human organs (Huebner et al. 2001). FRA3B is among the late-replicating regions that may not be completely replicated by the time the cell reaches G2 and mitosis, leaving the gaps often seen in metaphase chromosomes when cells are treated with aphidicolin, a mild DNA polymerase inhibitor. FRA3B was very frequently deleted in cancers suggesting some link between FRA3B and cancer initiation/progression. It became obvious that this gene was significant in the study of cancer and that it played an important role in preventing and suppressing cancer. For example, Fhit knockout mice show highly increased susceptibility to tumor induction and Fhit gene therapy can prevent or reverse tumor development (Pichiorri et al. 2008).

Loss of Fhit expression has observed in premalignant lesions of many organs in the body suggesting that the loss of Fhit expression plays a role in the initial stages of multistep carcinogenesis (for review, Pichiorri et al. 2009). Numerous studies of lung and breast cancers, for example, have shown that 50% to 90% showed reduced Fhit or lost expression. It was also found that loss of one Fhit allele in the Fhit knockout mouse strain could lead to development of tumors, showing that loss of one of the two Fhit alleles predisposes to tumor development (Huebner et al. 2001). The evidence is overwhelming that Fhit is somehow related to cancer development.

The protein itself is thought to be a tumor suppressor, which was first suggested from evidence of overexpression studies. Cancer cell lines that overexpress FHIT grew more slowly than their cancer-derived clones (Huebner et al. 2001). The Huebner laboratory also observed reduced tumorigenicity of FHIT-tranfected cells *in vivo*, suggesting that FHIT functions as a tumor suppressor gene both in vitro and in vivo. However Fhit signal pathways are important for

not just cancer cell growth control but also for normal cell growth control and maybe function through possibly more than one signal pathway (Pichiorri et al. 2008).

Paradoxically, examination of cells that have lost the FHIT gene product revealed that Fhit protein has functional roles in response to DNA damage (Halazonetis et al. 2008): 1) kidney epithelial cells established from Fhit-/- mice exhibited >2-fold increased chromosome breaks at fragile sites *vs* corresponding Fhit+/+ kidney cells (Saldivar et al. 2010); 2) the frequency of mutations following oxidative stress in Fhit-/- mouse embryo fibroblasts (MEFs) was nearly double that of Fhit+/+ MEFs (Turner et al. 2002). Despite these findings and the strong evidence that Fhit exerts tumor suppressor activity (Ishii et al. 2008; Jackson et al. 1998; Petermann et al. 2010; Sirbu et al. 2011; Lukas et al. 2011), it has been argued that deletions within the FHIT locus in transformed cells are passenger alterations rather than cancer-driving mutations (Harrigan et al. 2011). This study has further examined the role of Fhit loss in development of DNA damage and found that absence of Fhit causes genome instability.

Results

Fhit prevents spontaneous DNA damage

We hypothesized that Fhit may be involved in the prevention of, or the response to, DNA damage. To test this hypothesis we modulated Fhit expression in several cell lines, and assessed DNA damage using the neutral single-cell electrophoresis assay (Neutral Comet assay) and by Western blot analysis of the DNA damage marker, γH2AX. Each individual cell line was used because it is distinctly different from the others, allowing high confirmation that Fhit's role is consistant is all different types of cells.

The first cell line test we H1299 lunch cancer cells. These cells are cancerous therefore lack Fhit protein expression. To these cells, we inserted plasmids into the cells, E1 and D1 plasmids. The E1 plasmids were Fhit deficient so the cells remained lacking Fhit. The D1 cells however contained a Pon-A inducible segment that when treated with Pon-A, Fhit expression would be induced and the Fhit protein would now be present. Results from the neutral Comet assays revealed the presence of increased DSBs in Fhit negative (E1) H1299 lung cancer cells compared to the matching Fhit-positive (D1) H1299 cells. For example, the mean tail moment in E1 cells was 12.01 and the mean tail moment in D1 cells was drastically reduced compared to E1 cells (Figure 1c). Similarly, Fhit-negative (E4) MKN74 stomach cancer cells exhibited increased DSBs compared to the matching A116 clone (Fhit-positive MKN74 clone carrying stably transfected FHIT cDNA) (Figure 1b). To determine whether Fhit prevented DSBs in non-cancer cells, we tested kidney epithelial cells established from wild-type or Fhit knockout mice for DSBs using the comet assay. Fhit -/- kidney epithelial cells had increased mean tail moments compared to wild-type cells (Figure 1d). We also examined the occurrence of DSBs in noncancerous HEK293 cells. These cells already have Fhit expression so we treated them with

siRNA knockdown of Fhit expression and compared tail moments with the normal Fhit positive HEK293 cells. At 48 hours following Fhit knockdown, comet assays demonstrated increased DSBs in Fhit-depleted cells (Figure 1a), confirming that Fhit is involved in preventing DSBs in normal, transformed and cancerous cell lines. yH2AX is a protein that is expressed when there is DNA damage in the cell and it functions to mark DSBs in order to signal repair of the damage. Using western blots, E1 cells were observed to express more γH2AX than D1 cells, shown in Figure 1; thus, E1 cells exhibit increased foci of DNA damage.

Fhit Dinucleoside Substrate-binding activity is necessary for protection from DSBs

Once it was determined that Fhit was involved in preventing DNA DSBs, we investigated the mechanism whereby Fhit prevented this damage. Fhit possesses enzymatic activity, binding and hydrolyzing diadenosine triphosphate to form adenosine monophosphate and adenosine diphosphate. Fhit mutants that had altered enzymatic activity or reduced substrate-binding activity (Pichiorri et al. 2009) were used to determine if Fhit enzyme activity was required to prevent DNA breaks. We transfected the Fhit-negative H1299 cells with CMV expression plasmids with cDNAs encoding different Fhit mutants. The Fhit-H96N mutant binds substrate well but has little hydrolytic activity. The Fhit-Y114A has very poor binding affinity. The Fhit-Y114F mutant has weak binding affinity. Fhit mutant expression was analyzed by western blots to confirm that transfection with all the plasmids and expression of Fhit and Fhit mutant proteins was equally efficient. Immunofluorescence confirmed high transfection efficiency, ~80% for all mutants (Figure 2). Using the comet assay analysis we compared the level of DSBs present in cells expressing the different mutants. Expression of wild-type Fhit significantly reduced DNA damage, as expected, exhibiting an average tail moment close to 2 (arbitrary units). Y114A and

Y114F mutants did not reduce DNA damage relative to the Fhit-negative control, suggesting that the mutations altering substrate-binding affinity inactivate Fhit function. The H96N mutant modestly reduced DNA damage, but H96N mutant Fhit activity was diminished compared to wild-type Fhit. These findings indicate that the Fhit function to prevent DSBs depends upon substrate-binding and perhaps on catalytic activity.

Fhit prevents DNA replication stress

Endogenous DNA damage can be caused by DNA replication or oxidative stress. In a separate but related study in our lab, it was shown that Fhit does not prevent oxidative stress. This led us to hypothesize that Fhit might act to minimize events that induce endogenous replication stress. We examined the effect of different exogenously added agents that cause replication stress to study how Fhit-positive and Fhit-negative cells respond to replication stress. H1299 E1 (Fhit-negative) and D1 (Fhit-positive) cells were treated with three different drugs that induce replication stress, hydroxyurea (HU), aphidicolin (APH), and camptothecin (CPT). HU inhibits ribonucleotide reductase thereby depleting cells of the dNTPs needed for DNA replication and causing replication forks to stall and collapse. APH inhibits DNA polymerases slowing polymerase progression and causing forks to stall. CPT conjugates topoisomerase to DNA ahead of the DNA helicase, and thereby blocks the helicase from unwinding DNA and causing replication forks to stall. E1 and D1 cells were treated with HU, APH or CPT for 4 h and comet assays were performed to measure DNA breaks. For each inhibitor, E1 cells, which lack Fhit expression, DNA damage was unaltered compared to untreated cells (mean tail moments ranged between 10-12 for treated and untreated cells). In contrast, D1 cells, which express Fhit, had mean tail moments of 5, 12, 16 and 6, for the untreated, HU, APH, and CPT treated cells,

respectively. Thus, when HU or APH were added to D1 cells, the average tail moment increased to the level equivalent to the spontaneous tail moment in E1 cells (Figure 3). Thus, it is likely that Fhit prevents replication stress that is similar in nature to that induced by HU and APH.

Fhit and Chk1 activities are co-dependent

Cells have mechanisms to prevent, detect, and respond to replication stress. We considered proteins central to the DNA replication stress response and DNA damage response and assessed whether Fhit function was dependent upon their activities. The ATR/Chk1/ATM kinases are central to the S Phase checkpoint. Chk1 is a checkpoint kinase that responds to fork stalling during S/G2 phase during replication. It regulates the cell-cycle checkpoint to arrest cells in S and G2 phases. ATR plays a role at stalled forks and double strand breaks by activating Chk1 *via* phosphorylation of Chk1. ATM also acts by phosphorylating Chk1 in response to DSBs. While these 3 kinases have overlapping roles, they also perform separate specific functions. Knowing that Fhit prevents replication stress, specific small molecule inhibitors were used to inhibit each of these kinases individually in HEK293 and H1299 cells, and comet assays were used to measure DNA breaks. The results in Fig 4 show that Chk1 inhibition led to an increase in DSBs in siCtrl HEK293 cells. This increase was similar to the level of DNA breaks in untreated HEK293 Fhit-depleted cells. In contrast, Chk1 inhibition in Fhit knockdown HEK293 cells had little effect on DNA damage, suggesting that Fhit function to prevent DNA damage is dependent on Chk1 activity. ATR and ATM inhibitors were also used, but there was no dependency of Fhit function on either of these kinases. After it was confirmed that there was a functional interaction with Fhit and Chk1 in the HEK293 cells, we inhibited Chk1 in mouse

kidney cells and H1299 cells and observed similar results. Therefore, Fhit and Chk1 activities are co-dependent.

Figures and Charts

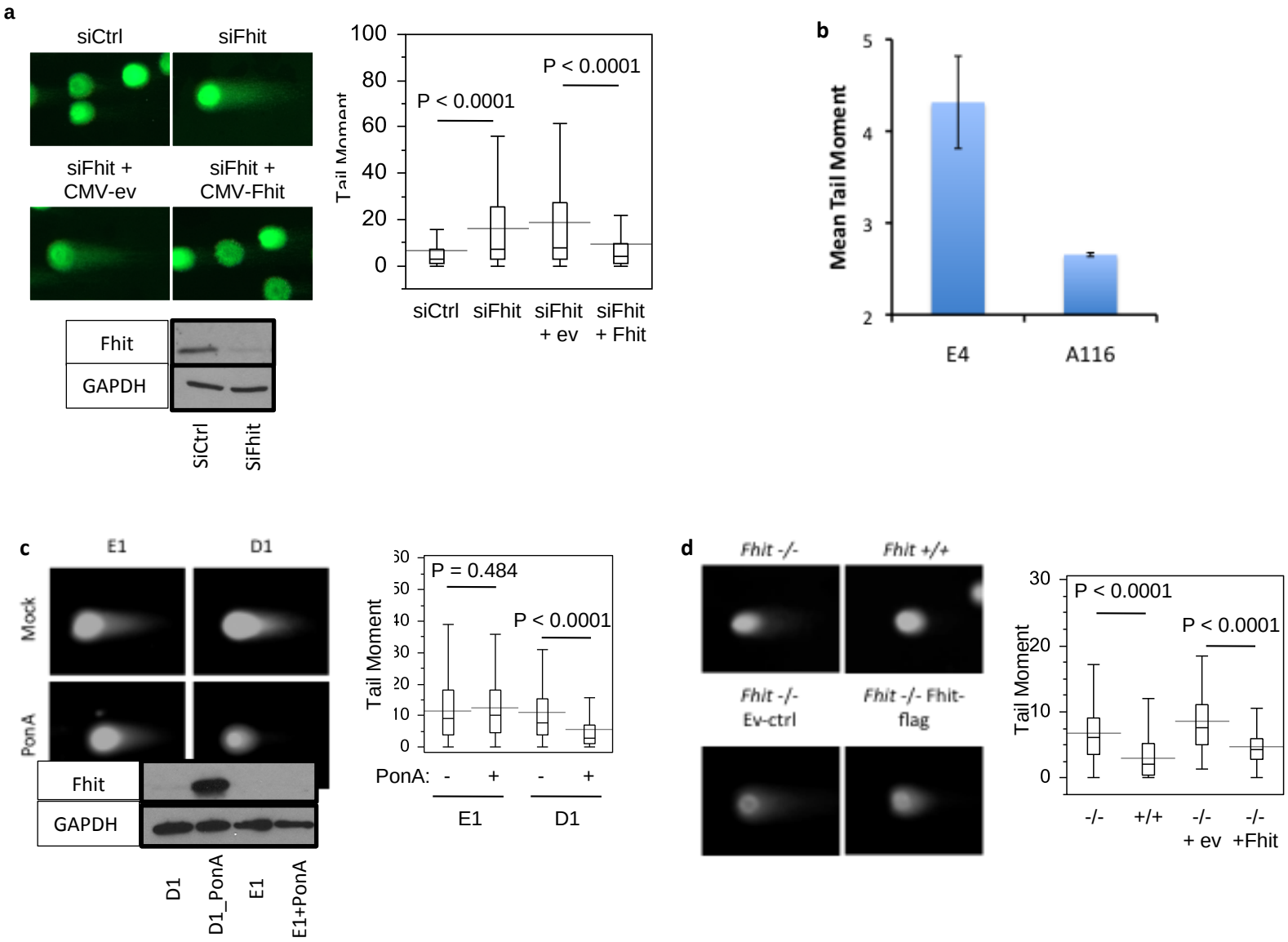

Figure 1: A comet assay and western blot were performed for each of the cell lines. A comet from each cell line is shown and the data is organized into a box plot. The top line depicts the highest tail moment recorded. The following data line, or top line of the box, represents the upper quartile. The middle line is the median and the bottom line of the box is the lower quartile. The line near the middle but not part of the box represents the average tail moment. The lowest line of the graph represents the smallest tail moment recorded. (a) A western blot and comet assay were performed on HEK293 cells with either siCtrl, siFhit, siFhit plus empty vector or siFhit plus Fhit expression vector. The western blot confirms there is significant siRNA knockdown of Fhit expression and the comet assay confirms that the Fhit knockdown siFhit cells demonstrated higher tail moments than the Fhit positive cells, thus more DNA damage. (b) MKN74 comet assay also confirms the A116 Fhit positive cells exhibit less DNA damage than the E4 Fhit-deficient cells (c) H1299 cells, naturally Fhit-deficient lung carcinoma cells, were previously transfected with an inducible FHIT cDNA and clones were isolated. These include the D1 clone (Fhit-positive) and E1 empty vector control clones. The western blot confirms that only D1+Pon A induced cells express Fhit. The comet assay confirms that the D1+PonA cells with Fhit expression exhibit significantly less DNA damage

than E1 cells. (d) The Mouse kidney cells demonstrated similar results with Fhit+/+ cells having less DNA damage than Fhit -/- cells.

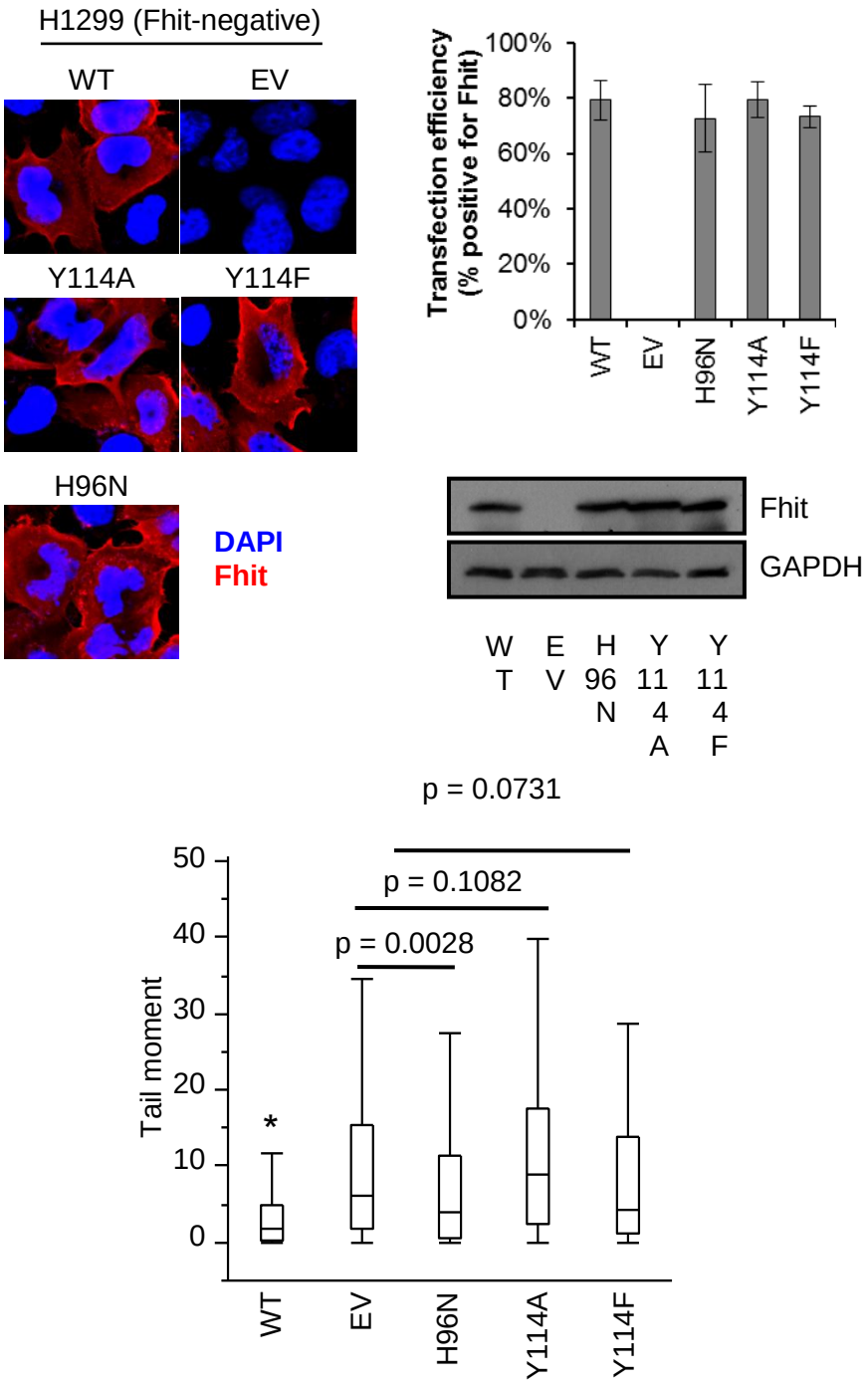

Figure 2: Western Blot performed on all mutants confirmed Fhit expression in all Fhit and mutant transfectants. Immunofluorescence confirmed successful transfection with an 80% transfection efficiency. Comet assay performed on mutants provided results suggesting that wild-type Fhit expression results in least DNA damage and empty vector transfection results in with the highest level of DNA damage. The three mutants exhibited levels of DNA damage similar to the empty vector transfected cells suggesting both enzymatic functions of Fhit are needed for sufficient Fhit function in preventing DNA DSBs.

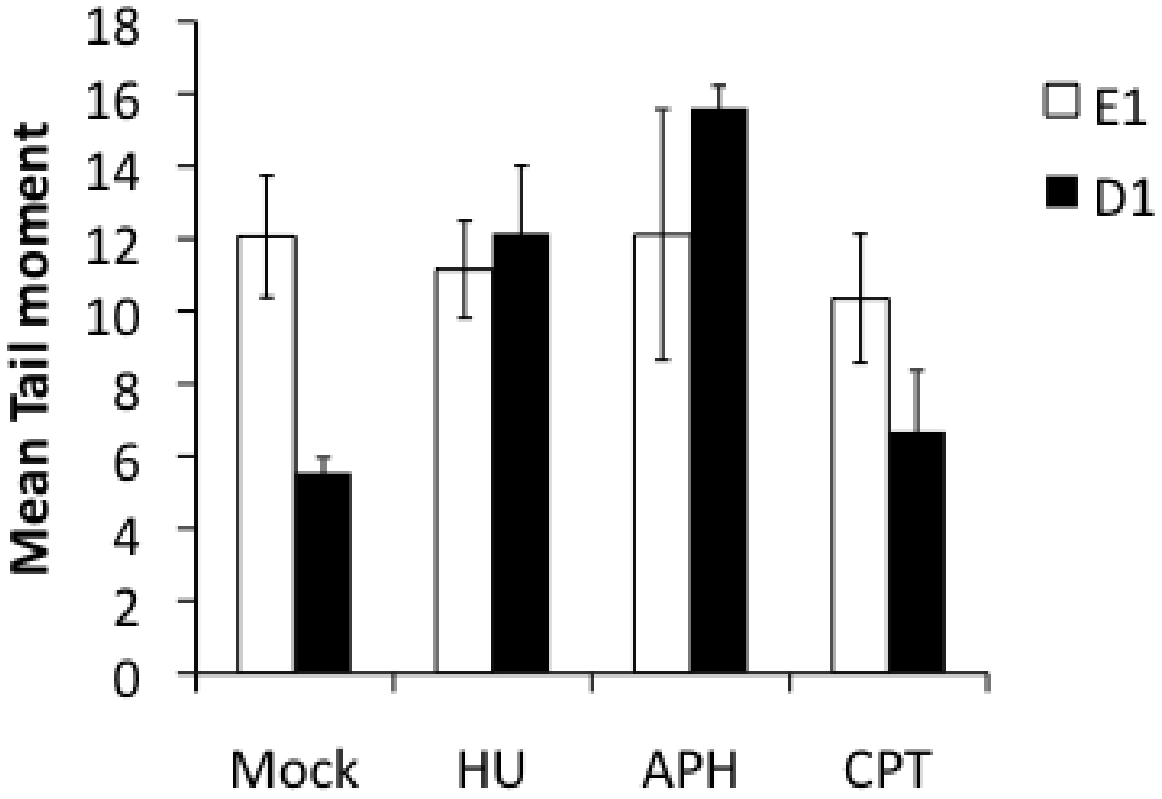

Figure 3: Comet Assay performed on H1299 E1 and D1 cells treated with HU, APH, and CPT. E1 cells exhibited similar levels of DNA damage/mean tail moments among the three treatments and the control mock E1. D1 results varied among the treatments with HU and APH treated D1 cells exhibiting high mean tail moments similar to the E1 cells. This trend was not seen in CPT treated D1 cells. This suggested that Fhit negative cells undergo the same type of stress induced by HU and APH treatment, but not similar to replication stress induced by CPT treatment.

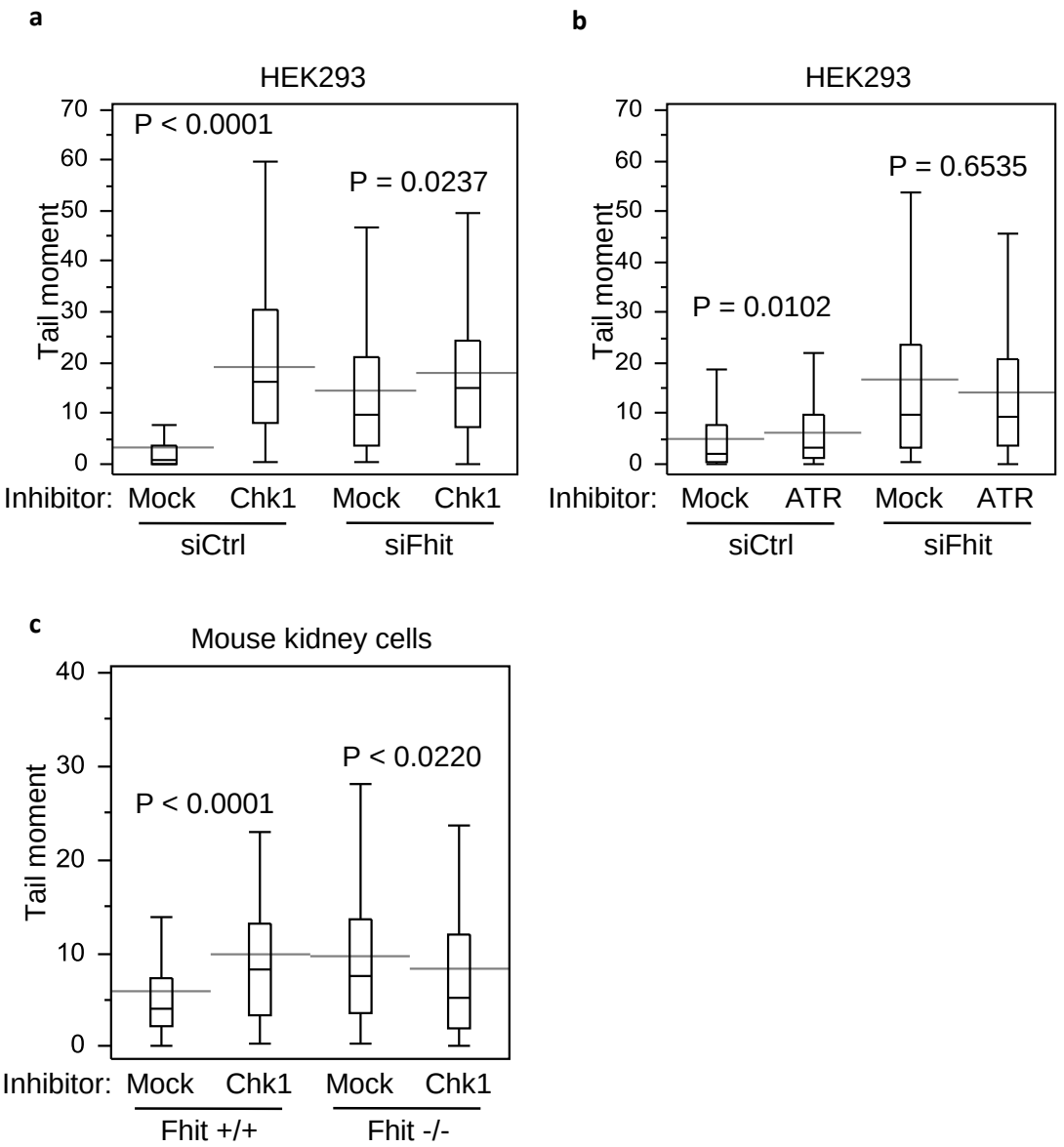

Figure 4: Comet Assays performed on HEK293 or Mouse Kidney cells treated with a kinase inhibitor: (a) HEK293 cells treated with 100 μM Chk1 Inhibitor for 2 h, Comet Assay performed and results show that in Fhit-depleted cells, there was little change in DSBs whereas we see an increase of DNA damage in Fhit positive siCtrl cells. (b) HEK293 cells treated with 50 μM ATR Inhibitor for 2 h. Comet Assay provides evidence that there is no change in DSBs with ATR inhibition in either Fhit positive or Fhit negative cells. Identical results were found with 10 μM ATM inhibitor treated HEK293 cells. (c) Mouse Kidney cells were treated with 100 μM Chk1 inhibitor to see results similar to those in (a). No change in Fhit negative but increase of DNA damage in Chk1 inhibited Fhit positive cells.

Discussion

For the first time, we have shown that Fhit plays a significant role in preventing DNA damage in many different types of cells lines including cancer, non-cancer, and mouse cell lines and no matter whether Fhit expression was silenced or induced. Studying Fhit mutants allowed us to find that the binding and cleaving of a dinucleoside substrate, or other *in vivo* substrate, by Fhit is a central mechanism for preventing DNA DSBs, since the mutants were not able to prevent the DNA DSBs. We were then able to show that Fhit acts to prevent double strand breaks caused from replication stress by comparing and examining the Fhit positive and negative cell reaction in different types of cell stress treatments. Knowing that Fhit is involved in the replication stress response, we then looked at possible interactions with other proteins involved in the replication stress response system to find that Fhit has a strong functional interaction with Chk1 kinase.

These findings have given us great insight into the role of Fhit as a 'caretaker' tumor suppressor. We know that cancer is caused by genomic instability and Fhit loss in many types of cancer has been observed. These results suggest that the loss of Fhit is a contributing factor to the initiation and progression of cancer because of the role Fhit plays in prevention of DNA damage. The fact that we see no DNA damage in the comet assay allows us to suggest that Fhit is in fact preventing as opposed to repairing DNA damage. If Fhit was repairing DNA damage, we would see evidence of DNA damage taking place in the comet assay, but we do not see this therefore it must act in DNA damage prevention. Fhit-deficient cells experience a significant increase in DSBs and these breaks can result in detrimental problems for the cell and ultimately increase genomic instability, making cancer initiation that much more likely. Knowing that Fhit is acting by binding or cleaving a dinucleoside substrate suggests that this substrate may also play an

important role in cancer development and genome stability. We can now move forward to ask more about the mechanism by which Fhit protects genome stability and what other players are involved in this mechanism.

We know that Fhit has a strong interaction with Chk1 but now we can focus on how these two are interacting. We can study further to find out if the interaction is direct or indirect and what type of interaction is taking place. Chk1 is a kinase that normally acts to phosphorylate its substrates, so possibly Chk1 is activating a protein *via* phosphorylation that allows this protein to work with Fhit to prevent DSB. Knowing that Fhit acts to prevent DNA DSBs and works somehow with Chk1 allows us to narrow down the future focus of studies of Fhit mechanisms of action.

This progress of understanding Fhit as a tumor suppressor gives us insight into applications for possible cancer prevention or treatment. In cancer cells, Fhit expression is lost and the cell develops DNA breaks. Cancer cells rely on DNA repair pathways to fix the DNA damage that occurs following Fhit loss. A Fhit-negative cancer cell would likely be more dependent on DNA repair pathways, thus using small molecule inhibitors that block DNA repair pathways, in combination with DNA-damaging therapeutic drugs, may be a strategy to selectively kill Fhit-negative cancer cells. Theoretically, normal cells would have several pathways for DNA repair and thus be less sensitive to DNA repair inhibitors. Studies such as this, employing a 'synthetic lethal' strategy, have been proposed and are in clinical trials, as reviewed in (Glazer et al. 2010); see the proposed model in the drawing below. For example, cells with mutant BRCA1, which encodes a protein needed for homologous recombination repair of DNA breaks, are selectively sensitive to PARP1 inhibitors, which inhibit alternative DNA repair pathways (cited in Glazer review). The underlying theory for this sort of synthetic lethal

strategy is: if you treat the cells in and around the tumor with a PARP inhibitor (protein involved in an alternative repair pathway), the normal cells will recruit BRCA1 to fix the DNA break, whereas the BRCA1-mutant cancer cells will have no other mechanism available to repair the damage and will inevitably undergo cell death. In the case of Fhit-negative cancer cells, one could imagine inhibiting a DSB repair protein (such as PARP) or a DNA damage response protein (such as ATM or ATR), and the Fhit-negative cancer cells would be sensitive due to increased spontaneous DNA breaks, while normal cells having minimal DNA breaks would be resistant. Further studies aimed to develop and test a therapeutic strategy based on the work of this study are of high importance.

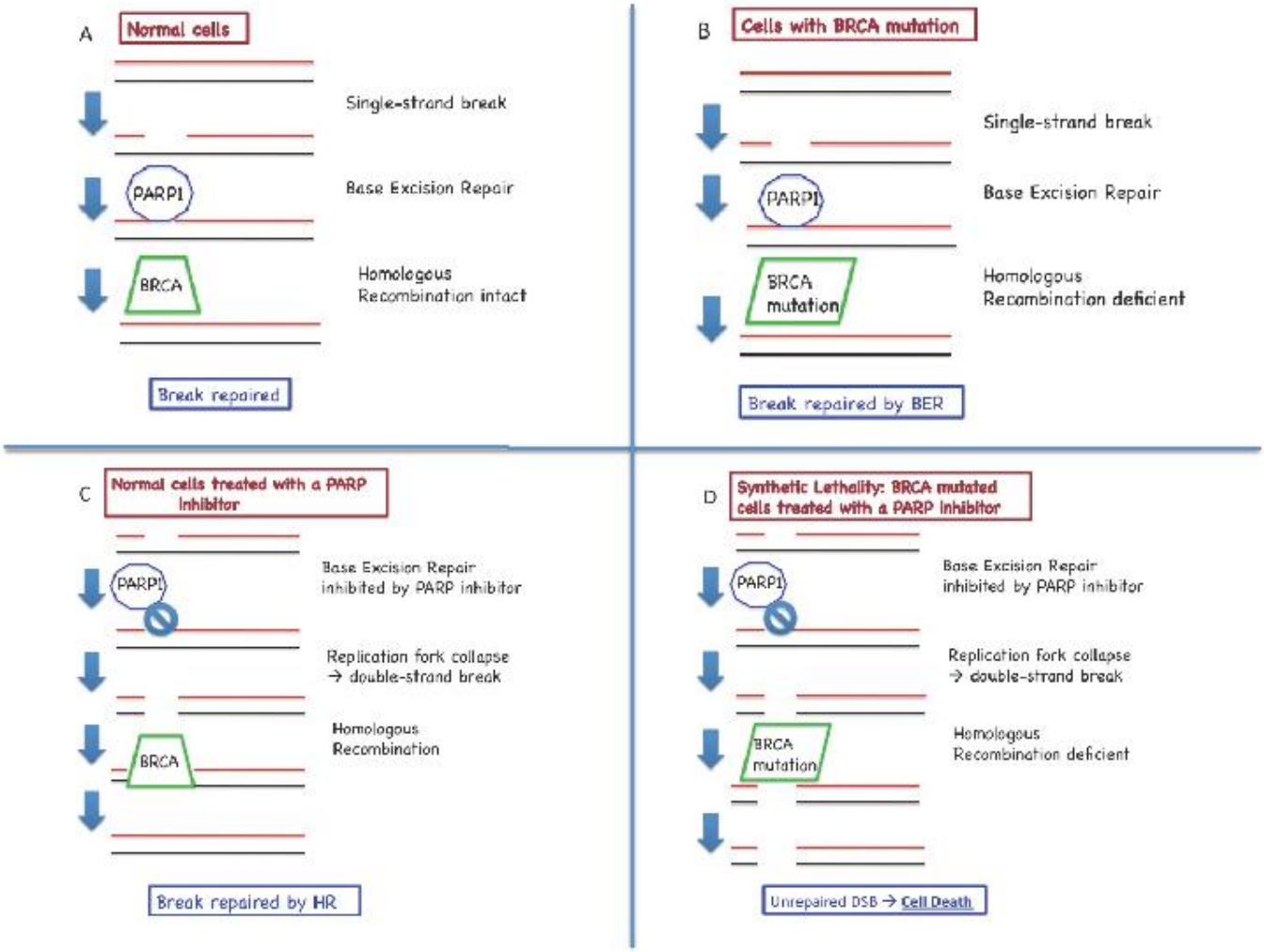

*Diagram taken from Glazer et al. 2010.

Methods and Materials

Cell Culture

E1 and D1 H1299 cells were cultured in MEM, 10% FBS, 100 μg/ml gentamicin, ziocin, and geneticin. HEK293 cells and Fhit+/+ and Fhit-/- mouse kidney cells were cultured in MEM with 10% FBS and 100 μg/ml gentamicin. Fhit negative MKN74 stomach carcinoma cells with either empty vector control (E4 cells) or exogenous Fhit expression (A116) were cultured in MEM with 10% FBS, gentamicin, and geneticin. All cell cultures were reseeded to prevent overcrowding and death in the culture about every two days.

Comet Assay

The protocol begins with removing cells from dishes *via* trypsin treatment. In the 60 mm petri dishes used in cell culture, about 0.5 ml of trypsin was used. Cells are placed in 15 ml conical tubes, washed with medium and centrifuged for 5 min at 600 RPM at room temperature. The pellet of cells is left undisturbed as liquid is aspirated out. The cells are then resuspended in 1 ml PBS or less depending on cell concentration. In separate 1.5 ml tubes, 10 ml of the PBS cell solution and 100 ml previously melted Agarose gel is added and mixed to immobilize cells. On the comet assay slides, 50 ml of this solution is added and slides refrigerated for 20 min. Once the gel has solidified, lysis solution is added and 4 degree incubation continued for 45 minutes to remove membranes and histones from DNA. Lysis buffer is aspirated and premade Electrophoresis Buffer (Water, Tris Base, Sodium Acetate) added to slides. Slides are kept cold for 25 min before placing in electrophoresis unit with the buffer. Settings should be 35 volts for 15 min for sufficient DNA damage to travel away from nucleus to create the “tail” in the assayed comets. After electrophoresis, slides are submerged in a premade DNA precipitation solution

(ammonium acetate, water, ethanol) for 25 min, then in 70% ethanol for 25 min. After both treatments, slides are dried, 100 ml SYBR Green I Stain added to each slide well for 30 min. Excess solution is allowed to slide off wells, with care not to touch the agarose itself. Once dry, the slide wells may be looked at through fluorescent microscopes and photos taken for analysis by computer program "CometScore." Tail moments are measured based on length and density of tail which correspond to amount of DNA damage and particularly DSBs.

Western Blot

After lysing cells, protein concentration was determined *via* Bradford Test and spectrometer. 25 µg protein were added to 1.5 ml tubes with lysis buffer and 4 µl 5xRSB together adding up to a volume of 20 µl. Tubes containing proteins were boiled for 5 min and cooled. Protein samples and 8 µl molecular weight rainbow marker were added into wells of gradient gel inside electrophoresis unit surrounded by ice at a voltage of 100. The proteins on the gel are transferred onto a membrane using the transfer unit at 45 volts for 15 min. Membrane is blocked for 1 h at room temperature in 5% milk solution (15 ml PBS, 0.75g dry milk). Primary antibody, rabbit anti-Fhit, rabbit anti-γH2AX, or mouse anti-GAPDH was added to membranes and incubated overnight at 4°C. Membranes were washed 3 x 10 min with PBST and secondary antibody mixed with 5% milk solution is added to membranes for 1 h at room temperature. Membranes are washed 3 x 10 min with PBST. A signal Pico solution was added to membranes with a 1:1 ratio of luminol and peroxide for 3 minutes then quickly plastic in plastic sleeves inside film cassettes. X-ray film was exposed to membranes in dark room and processed for visualization.

Immunofluorescence

Cells were grown on an 8-chamber slide and fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized with 70% ethanol for 20 min at 4°C, then blocked with 1% BSA for 30 min. Cells were treated with primary antibody, rabbit anti-Fhit, 1:500 in 1% BSA overnight at 4°C. Slides were washed 3 x 10 min in PBS, and secondary antibody was added, goat anti-rabbit IgG, 1:250 in 1% BSA for 1 h at room temperature in the dark. Slides were again washed 3 x 10 min PBS then a single drop of DAPI per chamber was added. Slides were covered and allowed to incubate for 1 h. Images were acquired with an Olympus FV1000 confocal microscope and analyzed using Image J software.

SiRNA Transfection

HEK293 cells ~75% confluent were transfected with siRNAs that target human FHIT or siRNAs that are non-specific control siRNAs. For every 60 mm^2 dish, 1 μg of siRNAs and 5 μL of Lipofectamine 2000 were added to 100 μl of Opti-MEM and incubated for 40 min. Cells were then washed in Opti-MEM and the siRNA/Lipofectamine solution was added. The cells were incubated for 5 h before 1.5 ml of normal medium with 2 x FBS solution was added. Cells were incubated at 37 °C overnight. Confirmation of siRNA knockdown of Fhit expression was by western blot analysis.

Cell Lysing

Lysis Buffer was previously made and mixed containing 980 ml lysis solution, 10 ml PMSF, and 10 ml CLAP. To a 60 mm dish, 50 μl of the lysis buffer is added to plates that then incubate on ice for 4 minutes. The cells were scraped from the plates and transferred to 1.5 ml tubes. The cell/lysis containing tubes were vortexed then allowed to incubate in ice for 10 min followed by

centrifugation for 10 min at 15000 RPM, 4 °C. The supernatant containing the soluble cell lysates are transferred to new tubes and frozen at -80 °C until needed.

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